Unmasking the Tonic Aversive State in Neuropathic Pain

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Supplementary Methods:

Animals: Male Sprague-Dawley rats weighing 250-275 g were employed in all studies. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Arizona, and were in accord with the US National Institutes of Health guidelines.

Surgical Procedures: *Intrathecal cannulation.* Rats were anesthetized with isofluorane and placed in a stereotaxic head holder. The atlanto-occipital membrane was exposed, cleared and an incision was made in the dura mater. A length of PE-10 tubing was advanced 8 cm caudally to the lumbar spinal cord. The tubing was exteriorized, filled with saline and plugged with wire. The wound was closed, and animals were allowed to recover for 7 days. Animals were not tested prior to the 7 day recovery period. Pilot studies revealed that testing prior to the 7 day recovery period resulted in limited number of crossings and resultant chamber bias, likely due to the invasiveness of the surgery. Drug administration was delivered in a 5 μl volume followed by a 9 μl saline flush. Progress was monitored with an air bubble. *RVM cannulation.* Rats were anesthetized with 100 mg/kg of a 10:1 mixture of ketamine and xylazine and placed in a stereotaxic headholder. The skull was exposed and two 26-gauge guide cannulae separated by 1.2 mm were directed toward the lateral portions of the RVM (AP –11.0 mm from bregma, L ± 0.6 mm; H–7.5 mm from the dura mater[1]). The cannulae were secured in place with screws and acrylic and the wound closed. As above, rats were allowed to recover for 7 days post-surgery. Microinjections were made by slowly expelling 0.5 μl of solution through a 33-gauge injection cannula inserted through each guide cannula and protruding 2 mm into fresh brain tissue. Progress was monitored with an air bubble. Upon termination of studies, pontamine blue dye was injected into the injection site for histological verification and only data from animals with verified to have correct cannula placement were used in analysis. *Nerve Injury.* Spinal nerve ligation (SNL) injury was produced by tight ligation of the L_5 and L_6 spinal nerves as previously described[2] and the spared nerve injury (SNI) was produced by tight ligation of the tibial and common peroneal nerves and sectioned distal to the ligation as previously described[3]. Sham control rats underwent the same surgery and handling as the experimental animals but without SNL or SNI. SNL or SNI was done 5-7 days following intrathecal or RVM surgery as pilot data demonstrated that doing i.th. and nerve injury surgeries at the same time impaired chamber crossings in baseline CPP measures (see below). Pilot studies revealed that testing prior to the 7 day recovery period resulted in limited number of crossings and resultant chamber bias, likely due to the invasiveness of the surgery. Following surgery the animals were allowed to recover for 7 days as specified above, at which time development of tactile hypersensitivity was verified. SNL or SNI rats that exhibit gross motor deficiency or failure to exhibit subsequent evoked pain (less than 10%) were excluded from further testing. Sham operated rats that exhibited tactile hypersensitivity (less than 5%) were excluded from further testing.

Behavioral observations.

Evaluation of Tactile Sensitivity. Rats were acclimated for 30 min in elevated cages with a wire mesh floor. A series of von Frey filaments (0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.5 and 15.1 g) were applied perpendicularly to the plantar surface of the hindpaw for 3 sec. A sharp withdrawal of the hindpaw indicated a positive response. The stimulus was incrementally increased until a positive response was obtained, then decreased until a negative result was observed in order to determine a pattern of responses to apply to non-parametric method of Dixon[4]. The 50% paw withdrawal threshold was determined as $(10[Xf+k\delta])/10{,}000$, where Xf = last von Frey filament employed, k = response pattern Dixon value, and δ = mean log-difference between stimuli.

CPP Procedures.

Multi-trial conditioning. Preconditioning to an automated 3 chamber CPP box was performed across 3 days, starting 7 days after surgery. All animals are exposed to the environment with full access to all chambers across 30 min each day. On day 3, behavior was recorded for 15 min and analyzed to verify absence of pre-conditioning chamber preference. Animals spending more than an 80% (time spent > 720 sec) or less than

20% (time spent < 120) of the total time in a chamber were eliminated from further testing (approximately 10% of total animals). Following the pre-conditioning phase, rats underwent conditioning across 6 days with alternating treatment-chamber pairings. Rats received vehicle (e.g. saline)-chamber pairing on odd days and drug (e.g. lidocaine microinjection)-chamber pairing on even days. Rats were placed in the paired chamber with no access to the other chamber immediately following vehicle or drug. Drug and chamber pairing were counterbalanced. On test day animals were placed into the neutral chamber, and had access to all chambers during the 15 min observation period, during which time spent in each of the chambers was recorded. Importantly, no drug was administered on test day, and so the time spent in each of the chambers was measured with the rat in a drug-free state. This is highly advantageous in that possible motor/sedative effects associated with drugs do not impact testing. Increased time spent in a chamber (e.g. increased time in the drug paired chamber) indicates preference for that chamber. Note that a corresponding decrease in time spent in the other chamber is observed (e.g. decreased time in the vehicle paired chamber).

Single trial conditioning. Starting 7 days post-SNL/sham surgery, all rats underwent a 3 day pre-conditioning period with behavior recorded on day 3 to verify no pre-conditioning chamber preference as described for the multi-trial conditioning. The following day (day 10 post-SNL), rats received the appropriate control (i.e. vehicle) paired with a randomly chosen chamber in the morning, and the appropriate drug treatment paired with the other chamber 4 hr later (afternoon). Chamber pairings were counterbalanced. On test day, 20 hours following the afternoon pairing, rats were placed in the CPP box with access to all chambers and their behavior recorded for 15 min for analysis for chamber preference. This protocol, in which vehicle was always paired 4 hr prior to the drug, was done to prevent potential confounds of long-lasting drug effects that may persist for longer than 4 hr (i.e., from the morning exposure). Such a case would interfere with the selective pairing of the drug effect with a specific chamber as the drug effect would be present in both chambers. In addition, pairing the drug with the chamber after the control treatment minimized the potential disruption in memory that could occur due to the handling and stress that may serve as a distraction during the second conditioning trial.

Note on pre-conditioning analyses: Analyses of the pre-conditioning (baseline) time spent in the conditioning chambers showed that rats spent equivalent levels in the striped vs. the black walled chambers indicating no pre-existing chamber preference prior to counterbalancing further suggesting that any post-conditioning preferences observed reflect preference due to relief of ongoing pain, and not other potential factors such as anxiolytic effects of drug administration.

Statistical Analysis: For experiments where evoked responses (i.e.; paw withdrawal thresholds and paw withdrawal latencies) were measured, behavioral data was collected prior to any treatment (baseline control values), and after SNL or sham surgery but before drug injections (post-surgery baseline) and at intervals of 15, 30, 45, 60, 90 and 120 minutes after drug administration. Differences over time between groups were determined by 2-factor ANOVA for repeated measures with time serving as a within-subject factor. Differences from the post- SNL or sham surgery values were determined by ANOVA followed by Student-Neuman-Keuls *post-hoc* test. For CPP experiments data was analyzed before conditioning (baseline) and after conditioning using two-factor ANOVA (chambers vs. treatment) followed by Student's t-test with Bonferroni correction. Statistical analysis for chamber preference prior to conditioning revealed no difference in time spent in chambers between sham and SNL treated rats for any of the experiments, therefore baseline chamber data for each experiment was pooled across surgical treatment. Difference from baseline scores were calculated for each rat using the formula: test time in chamber - preconditioning time spent in chamber. Difference from baseline scores for the drug paired chamber between SNL and sham operated rats were analyzed using paired t-tests. For all analyses, significance was set at $p<0.05$.

Supplementary References:

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4. Dixon, W.J. *Annu Rev Pharmacol Toxicol* **20**, 441-462 (1980).